A preliminary investigation of the utility of ribosomal genes for species identification of Sea Anemones (Cnidaria: Actiniaria)

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Citation: Worthington Wilmer, J. & Mitchell, M. L. 2008 12 01. A preliminary investigation of the utility of ribosomal genes for species identification of sea anemones (Cnidaria: Actiniaria). *In*, Davie, P.J.F. & Phillips, J.A. (Eds), Proceedings of the Thirteenth International Marine Biological Workshop, The Marine Fauna and Flora of Moreton Bay, Queensland. *Memoirs of the Queensland Museum — Nature* 54(1): 65–73. Brisbane. ISSN 0079-8835.

ABSTRACT

The utility of the ribosomal DNA gene complex for species identification of Actiniaria was examined. The use of universal ribosomal PCR primers is problematic in this group due to the presence of algal symbionts. Universal primers were initially used to amplify a region containing partial 18S, complete ITS, 5.8S, ITS2, and partial 28S sequences from six sea anemone species. The development of two sea anemone specific primers for this region was necessary to avoid amplification of algal symbionts for a number of species. Complete sequences of the 18S–28S fragment were obtained from three species, ?*Anemonia* sp. (724 bp), *Heteractis malu* (670 bp) and *Stichodactyla haddoni* (734 bp); partial or non-overlapping sequences were obtained from *Entacmaea quadricolor* (480bp from 18S), *Macrodactyla doreensis* (523 bp: 300bp from 18S and 223bp from 28S) and *Oulactis muscosa* (556bp: 285bp from 18s and 271bp from 28S). Average sequence divergence among sea anemone species was approx. 24% indicating that this region may indeed be useful for species identification. However, unexpectedly low divergence recorded between two species in different genera, neither of which could be verified by histology due to specimen unavailability, indicated that traditional histological methods are still needed to confirm identification and certainly until such time that an rDNA database of sea anemone tissue has been established. ❑ *ribosomal DNA; sea anemone specific primers; universal primers*

In February 2005, the Australian Marine Sciences Association, SEQ Branch, hosted the Thirteenth International Marine Biological Workshop, The Marine Fauna and Flora of Moreton Bay, Queensland. Fieldwork was conducted over a period of three weeks and occurred in a variety of environments including off-shore reefs, small islands accessible only at low tide, piers, estuarine mouths and mud flats. A taxonomic paper documenting the species found is presented by Fautin *et al.* (2008, this volume). Of the more than 20 species that are now known from Moreton Bay, we obtained tissue from the following six species and genera of anemones to assess the usefulness of DNA in identification, and the potential for understanding phylogenetic relationships: ?*Anemonia* sp., *Heteractis malu*, *Stichodactyla haddoni*, *Entacmaea quadricolor*, *Macrodactyla doreensis* and *Oulactis muscosa*.

Species identification of sea anemones (Anthozoa: Actiniaria) can be difficult, especially in the field. The taxonomic key currently utilised, designed by Oskar Carlgren (1949), is based mainly on histological differences and therefore requires

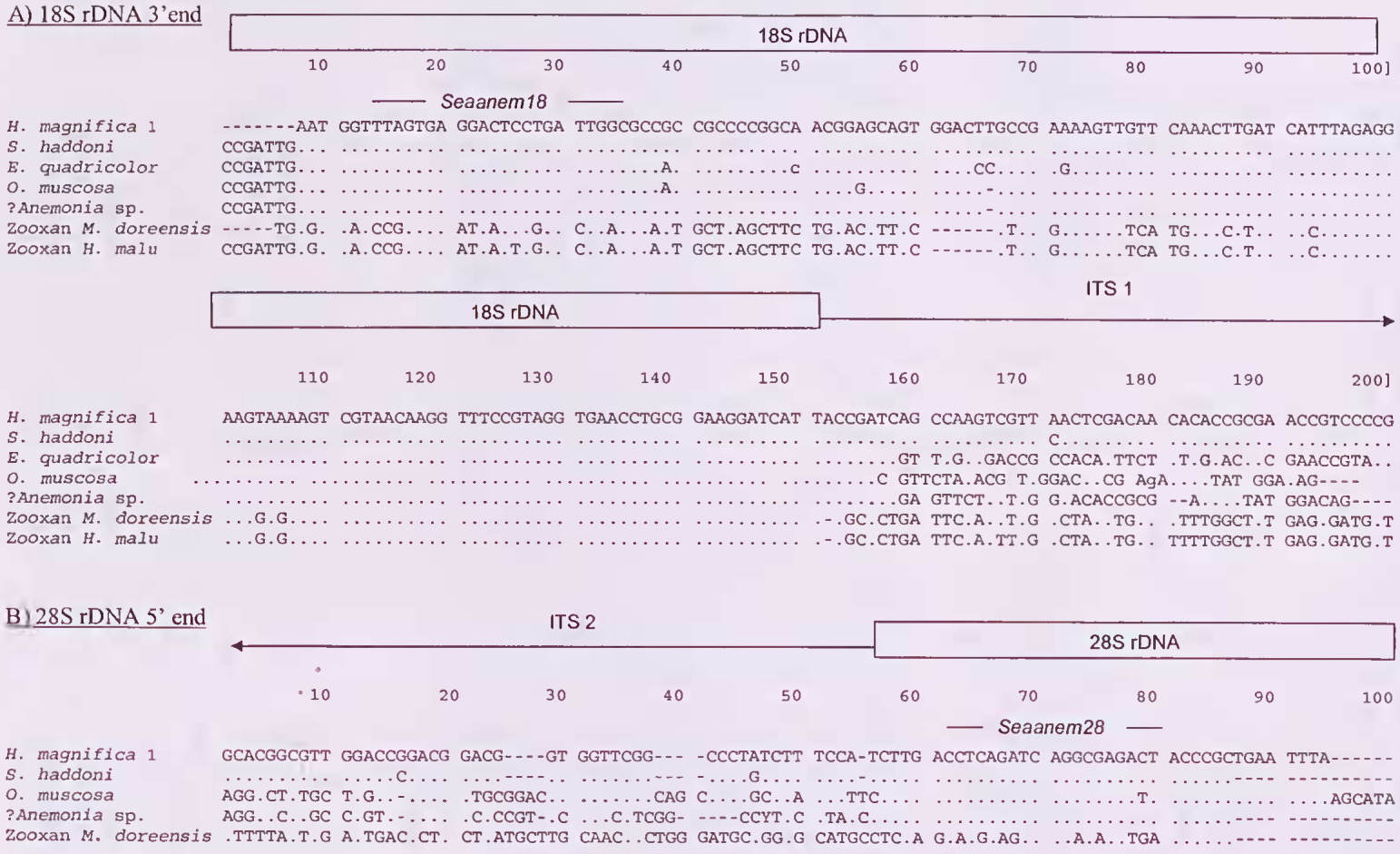

FIG. 1: Location of sea anemone specific primers Seaanem18S and Seaanem28S relative to a partial alignment of four anemones and two algal symbionts at **A**) 18S, and **B**) 28S rDNA genes. Intervening sequence between A) and B) not included. Sequence data for *H. magnifica* 1 was derived from GenBank (accession #AF050201).

Table 1. Moreton Bay sea anemone species used in the genetic analysis.

Collection Location	Latitude Longitude	Field Identification	Laboratory Identification	Genetic Source	Museum Reg. No.
Bird Island	27° 30′ S 153° 23′ E	*Entacmaea quadricolor* juv.	?*Anemonia* sp.	Whole animal	MTQ G58754
Flat Rock, N. Stradbroke I.	27° 24′ S 153° 33′ E	*Entacmaea quadricolor*	Not available	Tentacle	Whole spec. not coll.
Dunwich, flats in front of MBRS	27° 30′S 153° 24′E	*Stichodactyla haddoni*	Not available	Pedal disc & tentacle sample	Whole spec. not coll.
Shag Rock, N. Stradbroke I.	27° 24.85′S 153°31.59′E	*Heteractis malu*	*Heteractis malu*	Pedal disc & tentacle sample	MTQ G58749 QM Unreg.
Frenchmen's Beach	27° 25′ S 153° 32′ E	*Oulactis muscosa*	*Oulactis muscosa*	Pedal disc	MTQ G58756
Dunwich	27° 30′ S 153° 24′ E	*Macrodactyla doreensis*	*Macrodactyla doreensis*	Pedal disc & tentacle	MTQ G58748

collection of whole animals, which may not always be practical. Furthermore, histological analysis of sea anemones is time consuming and requires considerable expertise as some closely related species are almost impossible for the non specialist to identify, often resulting in incorrect taxonomic assignment (Stephenson 1928; Fautin 2000; Häussermann 2004).

Identification is further complicated by the fact that some species are virtually identical in appearance, distinguished by only one or two morphological features. For example, the two species *Heteractis malu* and *Heteractis crispa* are differentiated in the field on the basis of 1) column texture, which is firmer (leathery) in *H. crispa* than in *H. malu*, and 2) tentacle length, which is meant to be twice as long in *H. crispa* than *H. malu* (Fautin & Allen 1997). Both these characteristics can be misleading since the former is open to subjective interpretation if both species are not present side by side in the wild, while the latter may not necessarily be useful as the tentacles can be contracted at the time of collection/observation.

Alternatively, delineation of some species may be quickly achieved using appropriate molecular genetic methods (eg the Barcoding of Life initiative Hebert *et al.* 2003). However, previous genetic studies including sea anemone taxa have either only focused on questions pertaining to higher order anthozoan relationships (Won *et al.* 2001, Daly *et al.* 2003) or intraspecific population structure (Hunt & Ayre 1989). Numerous mitochondrial DNA genes such as COI (Fautin & Smith 1997), COIII and 16S rDNA (Geller & Walton 2001) have been used to infer phylogenetic relationships among the Actiniaria. However, mitochondrial gene sequence divergences within and among anthozoan families, including sea anemones, has been found to be significantly lower than other marine invertebrate species (Shearer *et al.* 2002). Barcoding studies also discovered that mitochondrial DNA evolved too slowly in sea anemones and other cnidarians for mtDNA differences to be an informative indicator of species (Hebert *et al.* 2003). Interestingly, Shearer *et al.* (2002) also found that, unlike all other metazoan taxa, substitution rates in anthozoan nuclear genes are much higher than in mitochondrial genes and therefore may be of greater utility in terms of species identification. Indeed, a number of other studies have suggested that the nuclear ribosomal (rDNA) gene complex incorporating 18S, ITS1, 5.8S, ITS2 and 28S could be ideally suited to examining below genus level relationships within the Actiniidae (McCommas 1991; Odorico & Miller 1997). Most recently Acuña *et al.* (2007) used the ITS region of rDNA in addition to morphology to distinguish between three species within the genus *Aulactinia*.

Molecular studies of sea anemones can be potentially complicated by the presence of symbiotic algae or zooxanthellae in the anemone tissue (Shearer *et al.* 2005) and possibly tissue

consistency (Pinto *et al.* 2000). If species possess zooxanthellae they are generally found in the gastrodermal tissues (i.e. tentacles and oral discs), although in some species they can be distributed heterogeneously throughout their hosts, being rare in only the pedal disc region or mesenteric tissue layers (Fautin & Smith 1997; Häussermann 2004). Therefore DNA extractions can contain both the host and algal genomes, which may cause confounding results especially for sequence data generated using broadly conserved or `universal' primers (see Shearer *et al.* 2005). A study by Pinto *et al.* (2000) found tissue consistency to impinge on the success of extraction of DNA from sea anemones, due to hardness of tissue from being preserved in ethanol. They concluded that a slow and gradual digestion method was optimal for extraction.

Here we conduct a preliminary study to examine the utility of the rDNA gene complex in the identification of sea anemone species and test whether a known universal primer pair is sufficient for such studies or whether anemone specific primers will be required. Furthermore we use modern DNA extraction kits to see if previous problems associated with sea anemone DNA extraction can be circumvented.

MATERIALS AND METHODS

SPECIMEN AND TISSUE COLLECTION

Collection techniques included; removing anemones from rocks by chisel and hammer, scraping animals off rocks by fingernail or taking a small tissue sample from the animal in the wild for genetic analysis if identification was 100% positive in the field. Tissues for analysis were collected from twelve species (based solely on field identifications). Of these, six samples representing an initial five species were used in the genetic analysis (Table 1). Additional samples of *Heteractis malu* were collected from Shag Rock subsequent to the workshop.

In order to examine and minimise possible zooxanthellae contamination, small tissue samples of less than 5mm in length were excised from either the lower column/pedal disc or, where possible, separate tissue samples from both the tentacles and pedal disc region of each species were taken. All samples for genetic analysis were stored in 100% ethanol. Where whole specimens were collected, tissue samples were taken after animals were relaxed in magnesium chloride and before being preserved in 10% formalin: seawater. All ethanol preserved tissue samples were stored at -20°C until genetic analyses were performed. *Heteractis malu* specimens collected subsequent to the workshop were stored in 100% ethanol and kept at room temperature (approximately 21°C) only.

DNA EXTRACTION, PCR AND SEQUENCING

To test to the usefulness of modern DNA extraction kits with ethanol preserved sea anemone tissues, total genomic DNA was extracted from both tentacles and pedal disc tissues using DNeasy Tissue Kits (QIAGEN) as opposed to the far more labour intensive protocol of Pinto *et al.* (2000). Partial 18S rDNA, complete ITS1, 5.8S, ITS2 and partial 28S rDNA sequences were initially amplified using the primer pairs RA2 and ITS2.2 described by Wörheide (1998) RA2 is located in the flanking 3' end of the small subunit ribosomal gene (18S) and ITS2.2 in the 5' end of the large subunit ribosomal gene (28S). PCR amplifications were performed in 25 μl reaction volumes and contained to a final concentration: 1x Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 μM each primer, 0.8 mM dNTPs and 0.75U of Taq polymerase. The use of the hot start polymerase HotMaster Taq (Eppendorf) required an initial denaturation at 94°C for 2 min prior to the commencement of the remaining cycle parameters; then followed 35 cycles of 94°C for 20 sec, 55–58°C for 20 sec, 65°C for 45 sec and a final extension 65°C for 5 min.

PCR products were gel purified using 'Perfect Prep' gel cleanup kit (Eppendorf) and forward and reverse sequencing reactions were carried out according to standard ABI PRISM dye-deoxy terminator sequencing protocols using Big Dye Terminator versions 1.1 and 3.1. Chromatographs were checked and all sequences were aligned using Se-Al v2.0a10 (Rambaut 1996). Estimates of sequence divergence including insertions (uncorrected p-distances) were calculated using the pairwise base distance function in PAUP* v4.0b10 (Swofford 2002). We verified the origin of the amplified sequence data by conducting a BLAST search in GenBank thus determining the phylogenetic affinity with sequences from other actiniarian or anthozoan species. Sequences

for this same region were also obtained from GenBank from two individuals of the species *Heteractis magnifica* (Accession no: AF050201 (*H. magnifica* 1) and AF050211 (*H. magnifica* 2)).

SEA ANEMONE PRIMERS

Based on the sequence results obtained from four of the six study species using the above described `universal' primers and one of the *H. magnifica* sequences plus contaminating zooxanthellae sequences from the remaining two species (*Heteractis malu* and *Macrodactyla doreensis* — see Results), we designed two new primers. These primers were designed to be specific to sea anemones and located in regions of identical sequence among the sea anemone species (for which we had data) but mismatched the zooxanthellae sequences at 45–50% of sites (see FIG. 1). These two new primers *seaanem18S*: 5′ TTA GTG AGG ACT CCT GAT TGG C 3′ and *seaanem28S*: 5′ AGT CTC GCC TGA TCT GAG G 3′ lie within 50bp downstream from RA2 and ITS2.2 respectively. We tested the primers against the same six species used with the `universal' primers. Amplification conditions, clean up and sequencing reactions with the new primers are identical to those described earlier.

RESULTS

DNA EXTRACTION

In contrast to Pinto *et al.* (2000) no problems were experienced extracting DNA from ethanol preserved sea anemone tissues using the DNeasy tissue kit. Prior treatment of the samples to remove ethanol was not required; nor did the tissues need to be homogenised in liquid nitrogen prior to the extraction process. Furthermore, total tissue digestion was completed within 1–3 hours at 55°C as recommended by the manufacture's protocol as opposed to the 72 hour period at 37°C used by Pinto *et al.* (2000).

UNIVERSAL PRIMERS

An 800bp (approximately) PCR fragment was successfully amplified from all six sea anemone species and all tissue types using the universal primers RA2 and ITS2.2. Readable sequence data of the fragment (including the 3′ end of the 18S gene, full length ITS1, 5.8S gene and ITS2 and the 5′ end of 28S gene) was obtained from only three of the six species (?*Auemonia* sp, *M. doreensis* and *S. haddoni*). Partial/non-overlapping sequences were obtained from the remaining three species (*E. quadricolor*, *H. malu* and *O. muscosa*). Not all tissue types generated readable sequence data. For example, sequences obtained from the pedal disc tissues of *H. malu* and *M. doreensis* were unreadable with evidence of multiple sequences present in the chromatograph (Table 2). This result was unexpected given that the amplified PCR product revealed a clear single band. However, readable sequence data was obtained from the tentacles of those same two species. BLAST searches of all readable sequences (either complete or partial) revealed strong matches (90–97% identity) with other sea anemone and/or anthozoan species in GenBank for only four of the six study species (Table 2). The sequence data obtained from the tentacles from *H. malu* and *M. doreensis* however, matched with almost 99% identity to other symbiotic algae sequences (e.g. *Symbiodinium* sp.) indicating preferential amplification of the zooxanthellae DNA in each of these species. Interestingly, the sequence data obtained from both the pedal disc and tentacles of *S. haddoni* were identical and BLAST searches of these and that obtained from the tentacles of *E. quadricolor* revealed closest similarity to other anthozoan species indicating that the host DNA had preferentially amplified and/or that zooxanthellae are either not present or in high enough density to mask the host DNA in both these species.

SEA ANEMONE PRIMERS

Amplification success using our primers *seaanem18S* and *seaanem28S* varied from that seen with the universal primers. Approximately 750 bp were obtained from five of the six anemone species; no PCR product amplified from *H. malu* regardless of tissue source (Table 2). For the three species for which either tentacle and/or pedal disc tissues were available, amplification success varied from species to species. No PCR product was obtained from *M. doreensis* tentacle DNA; in contrast, product amplified from the tentacle DNA of *E. quadricolor* and both tissue types for *S. haddoni* (Table 2).

The lack of amplification success for *H. malu* was surprising given that sequence data from the congeneric species, *H. magnifica*, was used in the alignment from which the new primers were designed and that the regions of both the

Table 2. PCR and sequence results obtained from anemone tissues using both the `universal' primers and sea anemone specific rDNA ITS primers. Presence (+) or absence (–) of product is indicated.

		Preferential amplification and sequence obtained using `Universal' primers		Preferential amplification and sequence obtained using sea anemone specific primers	
Species	**Tissue used in extractions**	**Anemone DNA**	**Zooxanthellae DNA**	**Anemone DNA**	**Zooxanthellae DNA**
?Anemonia sp.	Column / Pedal disc	+	–	+	–
Entacmaea quadricolor	Tentacle	+	–	+	–
Heteractis malu	Pedal disc Tentacle	+ –	+ +	– –	– –
Macrodactyla doreensis	Pedal disc Tentacle	+ –	+ +	+ –	– –
Oulactis muscosa	Column / Pedal disc	+	–	+	–
Stichodactyla haddoni	Pedal disc Tentacle	+ +	– –	+ +	– –

18S and 28S genes where these primers are located are identical among all the actiniarian genera (bar one site in *O. muscosa*), for which sequence data was available. In order to see if we could amplify a product for *H. malu* but avoid zooxanthellae DNA contamination, we tried the sea anemone primers in combination with the previously successful universal primers; using *seaanem 18S* paired with ITS2.2 and *seaanem28S* paired with RA2. Successful amplification from *H. malu* DNA from both pedal disc and tentacles was only obtained using *RA2/seaanem28S*.

Sequences, either partial or complete, obtained from *?Anemonia* sp., *O. muscosa* and *S. haddoni* using the new sea anemone primers were identical to those obtained using the universal primers, which had previously been confirmed as originating from host anemone DNA rather than their algal symbionts. BLAST searches of complete sequences from *M. doreensis* and *H. malu* obtained using anemone specific primers indicated greatest similarity to other anemones. Hence the anemone specific primers had been successful in circumventing the problems of zooxanthellae contamination. Curiously, *E. quadricolor* did not return readable sequence data suggesting that further optimisation of the sequencing reaction for this species and these primers may be required. For *H. malu*, sequence obtained with RA2 revealed no mismatches in the 3' region of the 18S rRNA gene where *seaanem18S* is located that would explain why this primer did not work on this species. Further experiments may be required to secure successful amplification with both anemone specific primers on this species.

In summary, complete or overlapping sequences of the 18S–28S fragment were obtained from only 3 species (*?Anemonia sp.* (724 bp), *H. malu* (670 bp) and *S. haddoni* (734 bp)). Although partial or non-overlapping sequences were obtained from *E. quadricolor* (480bp from 18S), *M. doreensis* (523 bp: 300bp from 18S and 223bp from 28S) and *O. muscosa* (556bp: 285bp from 18s and 271bp from 28S), they were excluded from subsequent analysis due to incompleteness.

SPECIES IDENTIFICATION

Among the three species for which full sequences were obtained (including the two *H. magnifica* sequences obtained from GenBank) estimates of sequence divergence ranged from 0.14% within *H. magnifica* up to 25.10% between *H. magnifica* 1 and *?Anemonia sp.* (Table 3). The average level of sequence divergence among species was 23.84% indicating that this region may indeed prove to be useful for species identification in sea anemones. The exception

was the comparison between *H. magnifica* and *S. haddoni*, where the divergence averaged only 1.7% (Table 3). This result was somewhat unanticipated given that it is significantly lower than the level of divergence found among the congeneric *H. magnifica* and *H. malu* sequences (ave 23.92%) and is therefore suggestive of possible taxonomic misidentifications. Considering that *H. magnifica* or *S. haddoni* cannot be taxonomically verified for this study due to specimen/tissue unavailability, it highlights the importance of using genetics in conjunction with traditional taxonomic methods.

The potential utility of this region for species identification is also evident from the example of ?*Anemonia* sp., which was tentatively identified in the field as resembling a juvenile *Entacmaea quadricolor* collected from Bird Island (Table 1). While only partial sequences were obtained from the adult *E. quadricolor* collected off Stradbroke Island, comparison of the sequences between the two specimens clearly showed they were significantly different (approx. 18% sequence divergence over 480bp) and possibly therefore two different species. Later histological analysis revealed that the the Bird Island specimen was not *E. quadricolor* as originally identified but may be ?*Anemonia* sp., although the exact identity of this species still awaits final taxonomic confirmation.

DISCUSSION

The ribosomal DNA gene complex has proved highly successful for species identification across an incredibly broad range of taxonomic groups including plants (Chase *et al.* 2005), fungi (Ristaino *et al.* 1998; Iwen *et al.* 2002), digenean parasites (Nolan & Cribb 2005) and mosquitos (Collins & Paskewitz 1996). It has even been used recently to identify commercial crustacean species from larvae collected in plankton surveys (Wang *et al.* 2006). In this study we investigated for the first time, the utility of this region for identification of sea anemone species and the potential problems of using universal primers in species, which contain algal symbionts.

While of a preliminary nature, our results showed high levels of sequence divergence among species using this region compared with divergence estimates an order of magnitude lower within a species indicating that it may indeed be ideal for assisting with sea anemone species identification. The questions at what taxonomic level and how useful this region may be for resolving phylogenetic relationships among sea anemone species was not the focus of this study but should certainly be investigated as more sequences become available. Acuña *et al.* (2007) used phylogenetic tools rather than estimates of sequence divergence to distinguish between different *Aulactinia* species and found extremely short branch lengths among individuals within a species compared to those between species.

The usefulness of conserved `universal' primers clearly depends on the species and tissue type available for analysis. However, as shown by the results obtained from *H. malu* and *M. doreensis*, extraction of `uncontaminated' host DNA from samples taken only from pedal disc tissues clearly should never be assumed. In order to guarantee that host DNA is amplified alone, use of primers specific to sea anemones are recommended; if not on their own then at least in combination with another universal primer. The extent to which the primers designed

Table 3. Estimates of sequence divergence among species for which the complete 18S–28S fragment was obtained (max 758bp). Sequences for *H. magnifica* obtained from GenBank. * Specimen collected subsequent to Workshop.

	?*Anemonia* sp.	*H. magnifica* 1	*H magnifica* 2	*H. malu*	*S. haddoni*
?*Anemonia* sp.	–				
H. magnifica 1	25.10%	–			
H. magnifica 2	24.93%	0.14%	–		
*H. malu**	21.60%	23.84%	23.99%	–	
S. haddoni	24.42%	1.64%	1.77%	22.98%	–

for this study will work across all actiniarians remains to be seen. Further preliminary PCR testing using *seaanem18S* and *seaanem28S* on another seven species from Moreton Bay, and representing another seven actiniarian genera, proved highly successful with strong amplicons produced in all seven species. Only subsequent sequencing will confirm whether or not the host DNA has been successfully targeted.

Modern DNA extraction kits also seem highly useful for overcoming any difficulties associated with DNA extraction from ethanol preserved sea anemone tissues. Why we experienced so few problems compared with the earlier work of Pinto *et al.* (2000) is unclear. It may be that we were able to work with tissues from recently ethanol preserved specimens, rather than ones, that had been in ethanol for an extended time.

Finally, a number of aspects of this study reinforce the value of being able to combine histological analysis with genetic testing to irrefutably verify a species' identity, especially given the embryonic stage of developing genetic markers for this group. In the case of a supposed juvenile *E. quadricolor*, the genetic data strongly indicated an incorrect field identification, and a subsequent histological analysis proved this to be so, identifying it instead as a probable *Anemonia* species. Furthermore, the curious result showing much greater sequence divergence between the two *Heteractis* species than that detected between *Heteractis magnifica* and *Stichodactyla haddoni* cannot, frustratingly, be resolved further. While again indicative of possible misidentifications, the *H. magnifica* sequences available on GenBank are not associated with registered specimens and the *S. haddoni* cannot be analysed histologically as the whole animal was not collected from the field.

ACKNOWLEDGEMENTS

We would like to thank Peter Davie and Darryl Potter for collection of the *H. malu* specimens used in this study subsequent to the workshop and Andrea Crowther for histological verification of some species. We would also like to thank P. Davie, D. Fautin and an anonymous reviewer for their insightful comments on an earlier version of the manuscript. M. Mitchell would like to thank the SE QLD Branch of the Australian Marine Sciences Association for the provision of the Scholarship to attend the Moreton Bay workshop, Dr Daphne Fautin for taxonomic training and Assoc. Prof. Peter Harrison and Dr Danny Bucher of Southern Cross University for their ongoing supervision and support.

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